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14. ABSTRACT

A 3-D model of primary human osteoblasts on a collagen scaffold was developed to study infection of osteoblasts with three Gram negative multi drug resistant bacteria, namely, Acinetobacter baumannii, Klebsiella pneumoniae and Pseudomonas aeruginosa. Post infection, osteoblast differentiation was determined in a temporal manner at 4, 24, 48 and 72 hours by gene expression utilizing real time PCR arrays. Alkaline phosphatase, Twist1, Dlx3, Dlx5, Stat 1 were differentially expressed post infection, suggesting that infection may affect differentiation and maturation of osteoblasts. Infection with all three pathogens resulted in proinflammatory chemokines IL-6 and IL-8 secretion. Once intracellular, all three pathogens may reinfect new osteoblasts. Intracellular infection also resulted in cytotoxcity to osteoblasts. Utilizing the 3-D model, the therapeutic effects of a well characterized antimicrobial peptide, KSL-W, was determined. KSL-W reduced the number of extracellular Acinetobacter baumannii and Klebsiella pneumoniae but not intracellular bacteria. The outcome of these findings will contribute to accelerate treatment options and management of osteomyelitis due to drug resistant bacteria.

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INTRODUCTION

The developmental processes for bone tissue is complex and involves several tightly regulated gene expression patterns of bone associated proteins. The expression of these genes and proteins are regulated in a temporal manner both in vivo and in vitro and has previously been studied in both these systems. The process of osteoblast differentiation can be subdivided into 3 stages, namely, proliferation, extracellular matrix synthesis and maturation and, lastly, mineralization.

Each stage is characterized by the expression of distinctive bone markers. It has been demonstrated by Stein and Lian [1] that during bone formation the expression of extracellular matrix genes (type I collagen, fibronectin, transforming growth factor-131), cell cycle or growth-related genes (histone, c-fos, c-myc) increased initially, followed by genes associated with mineralization, such as osteocalcin, osteopontin and bone sialoprotein. In addition, osteoblastproduced circulating cytokines and growth factors also influence the differentiation process.

We proposed to determine early in vitro gene expression changes in osteoblasts when infected with multidrug resistant Gram negative bacteria. An in vitro 3 dimensional (3-D) model of primary human osteoblasts on a collagen scaffold was to be developed for the study. The 3-D osteoblast cultures would then be infected with clinical isolates of multidrug resistant *Acinetobacter baumannii* (AB), *Klebsiella pneumonia* (KP) and *Pseudomonas aeruginosa* (PA). Post infection, early osteoblast differentiation and maturation processes would be determined in a temporal manner by gene expression. Utilizing this model, the therapeutic effects of antimicrobial peptides on osteoblasts infected with these three bacteria would be determined.

BODY

Milestone 1: In vitro infection studies

A collagen scaffold model with primary human osteoblasts was developed in the laboratory following modifications of Shen et al [2]. A working model was first established with the human osteosarcoma cell line SaOs2. The first year's work was based on the establishment of the 3-D collagen scaffold model with osteoblast cell line SaOs2 for infections. Work was completed successfully to establish the model, and infections were performed with clinical isolates of multidrug resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with SaOs2 cells prior to working with primary osteoblasts.

In the second year, work began with primary osteoblasts utilizing the established 3-D collagen scaffold model. In order to relate to war trauma, primary osteoblasts from healthy male subjects between the ages of 18-45 years were purchased from commercial sources (Lonza and Promocell Inc). In the third year, work was completed on gene array studies (n=3). Utilizing this model, the peptide KSL-W was tested against all 3 pathogens for bactericidal activities.

Accomplished work for Milestone 1:

1.1 Establishment of a 3-D osteoblast model in vitro

Initial work was performed with human osteoblast cell line SaOs2. Osteoblasts were seeded on collagen scaffolds [Fig 1]. Two methods of seeding were compared (Drop-IN and Drop- ON) [Fig 2] and the Drop-IN method was utilized for further studies. Osteoblast viability on 3-D scaffolds was determined by trypan blue exclusion [Fig 3]. Osteoblast marker expression was used to evaluate the differentiation of osteoblasts on the collagen scaffold. Cells were assessed for the expression of osteoblast markers, namely, collagen 1, osteocalcin and alkaline phosphatase (ALP) over time [Figs 4, 5 and 6]. These markers are for early and late osteoblast differentiation and maturation. Results indicated the presence of maturation and differentiation markers increased over time in SaOs2 cells on the 3-D collagen scaffold, and the quantity of ALP in osteoblasts increased over time [Fig 6]. From these studies, it was determined that the cells in the scaffolds were differentiating over time. A time point between 11-14

days was selected for infections as it showed increasing ALP, collagen 1 and osteocalcin expression in cells, indicating differentiation and matrix maturation of osteoblasts. Once the scaffold model was established, infection with bacteria was performed.

In the past, others have developed 3-D scaffolds with osteoblasts in vitro for bone regeneration [3], [4], [5]. Recently, investigators have reported infections utilizing a collagen-osteoblast model [6].

1.2 Infection of primary osteoblasts in the 3-D model (invasion assays)

Clinical isolates of MDR *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were obtained from WRAMC. For infections, bacterial cultures from an LB agar plate streaked overnight were used. Bacteria were re-suspended in saline. OD's and appropriate CFUs were estimated for infections. As all three species of bacteria have been reported to invade epithelial and other cells [7, 8, 9] we investigated if these pathogens could also invade osteoblasts. Primary human osteoblasts seeded on scaffolds for days 11-14 were used for infections. The day before infection, media was replaced in wells with antibiotic free media. For infections, excess media was removed on sterile Kimwipe from each scaffold and moved to a 6 well plate. The appropriate number of bacteria was added onto the scaffold in a 10ul volume. Scaffolds were incubated for 2hrs at 37^0C. After 2 hours, each scaffold was rinsed in PBS and placed in a 24 well plate with DMEM + 10% FBS and 100ug/ml polymyxin B. After 1 hour (t=0) for extracellular bacteria to be killed by polymyxin B, and at each time point, scaffolds were rinsed in PBS, and digested in 1mg/ml collagenase for 30 minutes, and cell pellets were obtained after centrifugation at 2500rpm for 2 minutes in a micro-centrifuge. At each time point, cells were lysed with deionized distilled water. Cell lysates were plated on LB agar plates and the CFUs were enumerated the following day. Supernates from each well were plated out to ensure killing of extracellular bacteria (data not shown). Invasion assays performed on osteoblasts in the collagen scaffolds indicated that all 3 species of bacteria could be detected intra-cellularly [Fig. 7].

The fate of these Gram negative intracellular pathogens after the death of the osteoblast is not known. The ability of released intracellular bacteria to re-infect new osteoblasts was investigated. In the invasion assays, to enumerate intracellular bacteria in osteoblasts, deionized water was used to release bacteria. This method, however, does not mimic the natural process of death of osteoblasts. We plated infected whole osteoblasts (not lysed) in order to look at the fate of intracellular bacteria after a more

natural death of osteoblasts. Recovered bacteria were then used to re-infect new cells, thus creating a process which could reflect the clinical situation.

Monolayer SaOS2 osteoblasts were infected with AB, KP or PA. Infection proceeded for 2 hours. After 2 hours, wells were rinsed with PBS and media containing 100ug/ml of polymyxin B was added for 1 hour to kill extracellular bacteria. At 0, 24 and 72 hours post infection, infected cells were either lysed with distilled deionized water, or whole unlysed cells were plated on agar plates after the addition of Trypsin-EDTA to release cells from tissue culture plate. After overnight incubation of plates at 37^0C the bacterial CFU's recovered from whole cells on agar plates were used to re-infect fresh SaOs2 cells. We found that that lysis of osteoblasts is a better method to recover intracellular bacteria compared to plating whole cells [Table 1]. The differences in CFU may be due to less survival of bacteria in dying host cells. Alternatively, intracellular bacteria released from dying host cells are not dispersed evenly so that the total CFUs detected may be less. Our results suggest that all three species, once released from dying osteoblasts, have the ability to re-infect healthy cells [Table 2].

1.3 Cytotoxicity assays for infection of osteoblasts with Acinetobacter, Klebsiella and Pseudomonas sp
In order to determine if intracellular infections with these three pathogens would result in cytotoxicity to osteoblasts, LDH (lactate dehydrogenase) assays (Cytotox 96 cytotoxicity assay Promega, WI) were carried out on infected osteoblasts on 3-D scaffolds. This assay quantitatively measures LDH, a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernates is measured coupled enzymatic assay which results in the conversion of tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of lysed cells as detected using a plate reader. Infections were performed on primary osteoblasts on 3-D scaffolds according to the manufacturer's instructions (Promega). LDH levels were determined from a standard curve. The highest toxicity was detected in osteoblasts infected with *Klebsiella pneumoniae*. Higher levels of osteoblast toxicity were observed at each time point after infection with all 3 bacteria compared to uninfected control [Fig 8].

1.4 Studies on Osteoblast gene expression during infection with Acinetobacter baumannii, Klebsiella pneumoniae and Pseudomonas aeruginosa. RNA extractions of osteoblasts on 3-D scaffold for gene expression

Primary human osteoblasts at 3-4th passage on confluent flasks were harvested and scaffolds loaded as described previously. On day 12-13, infections with AB, KP and PA were performed as described previously. The MOI for each bacterial species was established as 100:1 for *Acinetobacter baumannii* 5075, 20:1 for *Klebsiella pneumoniae* and 50:1 for *Pseudomonas aeruginosa*. Scaffolds were incubated for 2hrs at 37^{0}C. After 2 hours, each scaffold was rinsed in PBS and placed in a 24 well plate containing media with 10% FBS and 50ug/ml ascorbic acid and 100ug/ml polymyxin B. Infected primary osteoblasts on 3-D scaffolds were collected at 4, 24, 48 and 72 hours post infection. A set of uninfected control osteoblasts were included at each time point. 4-5 scaffolds at each time point were pooled together for extraction of total RNA.

Scaffolds were digested with 1mg/ml collagenase, and a cell pellet was obtained after centrifugation at 2000RPM. 1ml of RNAzol was added to each pellet and re-suspended. The samples were frozen at -80^{0}C until used for the gene expression study. Total RNA was extracted with the Qiagen miniprep kit (Qiagen, Inc.). RNA was verified for concentration and purity by Nanodrop and Agilent 2100 Bioanalyzer. From the literature, a panel of genes involved in osteoblast differentiation and maturation were selected, and a custom real time PCR array was designed (Qiagen, SA Biosciences) to probe the infected/control samples. The list of genes for the custom array is shown in Table 3.

Total RNA from primary osteoblasts infected with AB, KP and PA at t=4, 24, 48 and 72 hours post infection were used to probe the arrays. An uninfected control was included at each time point. The PCR Arrays consisted of sets of optimized real-time PCR primer assays with osteoblast differentiation and maturation focused genes as well as appropriate RNA quality controls on 96- well plate format. The RT PCR arrays performed gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray.

The RT PCR array runs were completed with RNAs from osteoblasts following the manufacturer's instructions (QIAGEN RT^2 Profiler^{TM}). Briefly, total RNA (500ng) isolated from osteoblasts were converted to cDNA according to the manufacturer's protocol (Qiagen Inc). cDNA was added to RT^2 qPCR master mix, and was added to the 96 well custom array which included the appropriate primers for each gene. PCR arrays were then run in an ABI 7000 instrument. Data was uploaded according to instructions. The integrated web-based software package for the PCR Array System provided by Qiagen

(SA BioSciences) automatically performed all ΔΔCt based fold-change calculations from the uploaded raw threshold cycle data. The mRNA expression levels obtained for each gene were normalized to the mean expression of GAPDH and beta actin housekeeping genes by using the following equation: relative mRNA expression = $_2$−(Ct of test gene −Ct of mean housekeeping genes) (where Ct is the threshold cycle). Up-regulated and down-regulated gene expression fold changes were represented by positive and negative values respectively. Statistical significance of each expressed gene was performed using Students t-test.

Alkaline phosphatase (ALP) is a biomarker of bone formation and active osteoblasts have high expression of ALP. ALP is involved in osteoblast maturation, and it is a marker for bone metabolism; in vitro studies have shown that expression of ALP increases with the differentiation of osteoblasts [1]. Early in the developmental sequence of the osteoblast, at one point, proliferation ceases and expression of genes related to the differentiated phenotype of osteoblasts is initiated. ALP has been found in differentiation studies with osteoblast-like cell lines in vitro [1]. In our study, when osteoblasts were infected with AB, ALP levels were down-regulated except for 24 hours post infection when induction of ALP was seen. For KP, ALP levels were down-regulated at 24 hours. PA infection resulted in the down-regulation of ALP at 48 and 72 hours post infection [Table 4] suggesting that osteoblast differentiation/maturation was decreased at one or more time points when infected by any of these 3 wound pathogens. The implication for this observation would be that local delivery of some antibiotics has been reported to decrease the levels of ALP in healthy osteoblasts [10]. As ALP levels are also down-regulated by infection, it may be important to carefully consider appropriate antibiotics to treat bone infections due to any of these 3 pathogens.

Osteocalcin (BGLAP) constitutes the most abundant non-collagenous protein present in bone and an extracellular matrix protein [11]. Serum levels of osteocalcin correlate closely with bone formation [12] and, this marker has been widely used as an indicator of new bone formation in vivo [13]. Osteocalcin production correlates with the onset of bone mineralization [14]. BGLAP synthesized in the bone almost exclusively by the osteoblasts has been considered as a bone formation marker. Our data shows that osteocalcin expression was decreased at some time point for all 3 pathogens during the course of infection. The lower expression levels of both ALP and BGLAP bone markers during infection at some

time point suggest that the intracellular presence of these bacteria in osteoblasts (though in small numbers) may affect the normal differentiation and maturation of osteoblasts.

A number of transcription factors are accountable for the regulation of osteoblast differentiation and function. These include Runt-related transcription factor (Runx2), Distal-less homeobox 3 (Dlx3), Distal-less homeobox 5 (Dlx5), msh homeobox 2 (MSX2) and osterix (Sp7) among others, and were investigated for mRNA expression during infection.

Runx2, a cell-specific member of the Runt family of transcription factors, plays a critical role in cellular differentiation processes in osteoblasts [15] as the crucial osteogenic transcription factor expressed in early bone development. It also persists through different stages of bone formation [15, 16]. RUNX2 expression was similar to uninfected cells for all time points post infection with AB, KP or PA, except its expression increased 3.49 fold at only 72 hours when infected with PA [data not shown]. A second transcription factor downstream of RUNX2 is Sp7 and it remained unchanged during infection to PA and increased at 24 hours for AB and 72 hours post infection to KP [table 5] suggesting that infection did not decrease any osteogenic activities of RUNX2 and Sp1.

Dlx5 is a bone inducing transcription factor which plays an important role in osteoblast differentiation that is expressed in the later stages [17] suggesting that Dlx5 may be involved in the maturation of the bone cell phenotype [18]. We saw that Dlx5 expression was down-regulated with AB infection, and also for PA suggesting a role in maturation delay of infected osteoblasts. Infection with KP did not alter the expression of Dlx5.

A role for Dlx3 in the up-regulation of bone-related genes to promote osteoblast differentiation has been found and shows that Dlx3 may enhance osteoblastogenesis [18]. It was also found that both Dlx3 and Dlx5 expression overlap in part during the matrix maturation stage. During osteoblast differentiation, both Dlx3 and Dlx5 act together to control the expression of osteocalcin gene. Therefore, findings suggest that the Dlx proteins may function in a complex regulatory pathway towards osteoblast differentiation. During infection with AB, KP or PA, we saw that the expression of Dlx3 gene was also down-regulated at some time points compared to uninfected cells, again suggesting that intracellular AB, KP or PA may affect the processes of osteoblast differentiation and maturation.

The transcription factor MSX2 was significantly induced to AB infection at 24 hours. Both KP and PA infections had no response to MSX2 (data not shown). Studies have demonstrated that Msx2 promotes osteoblast differentiation and/or proliferation [19, 20]. Yet, another study by Liu et al. [21] suggested that Msx2 inhibits differentiation of osteoblast precursors and immature osteoblasts.

Twist 1 is an essential transcription factor, whose down-regulation is required for osteoblast differentiation. Studies have revealed that the role of Runx2 in osteoblast differentiation was controlled by Twist1. Twist1 is able to inhibit osteoblast differentiation without affecting RUNX2 expression [22]. Even though infection with KP did not result in changes in Twist1 expression, its up-regulation in AB and PA infections [Table 6] suggest that differentiation of osteoblasts may be compromised when infected with AB and PA in the first 72 hours of infection.

In bone biology, studies have revealed that Signal transducer and activator of transcription 1 (Stat1) is involved in osteoclastogenesis and osteoblast differentiation in addition to immune regulation [23]. Previously, it was observed that osteoblast differentiation in vitro was significantly enhanced in the absence of Stat1 indicating that Stat1 interferes with osteoblast differentiation. Also, Stat1 acts as an attenuator of Runx2 in the cytoplasm [23]. Stat1 has been found to be a negative regulator associated with both bone formation and resorption and it maintains the homeostasis of the skeletal system. Our data indicates that when infected with AB, KP or PA, Stat1 was up-regulated at several time points, suggesting that osteoblast differentiation may be obstructed due to the activation of Stat1 during infection with AB, KP or PA.

Among growth factors involved in osteoblast differentiation, bone morphogenetic protein (BMP) family proteins play a crucial role in osteogenesis. BMPs are members of the transforming growth factor-13 (TGF-13) superfamily and regulate osteoblast function and development and are vital to this intercellular signaling system, and they activate osteoblast differentiation. Studies with recombinant BMP's 2, 4, 6, and 7 have shown that they are strong inducers of osteoblast differentiation. In addition, BMP-2 and BMP-7 induce the activation and expression of Runx2 [24-27]. BMP-2 stimulates osteoblast differentiation and bone formation [27-30]. We observed the induction of BMP-2 in only AB infected osteoblasts suggesting that it promoted proliferation and differentiation during infection with AB, and

BMP-7 was induced in both AB and KP at different times. PA infection did not affect the expression of either gene [Table 7].

Cell–cell adhesion by cadherins is essential for the function of bone forming cells during osteogenesis. Also, cell adhesion is associated with other biological processes such as the immune response, wound healing and tissue structure maintenance. Osteoblasts express a limited number of cadherins, including the classic N-cadherin, CDH2. The expression profile of N-cadherin in osteoblasts during bone formation in vivo and in vitro suggests a role of this molecule in osteogenesis. Our data shows that both KP and PA induced CDH2 at 4 hours post-infection, but by 72 hours, it was down-regulated. When infected with AB, CDH2 was down-regulated at 48hours [Table 8]. It has been found that interruption of cadherin-mediated adhesion prevents induction of a fully differentiated osteoblastic phenotype [31]. We found that CDH4 was not significantly expressed to any of these pathogens except for the down-regulation at 24 hours after infection with KP.

When osteoblasts are activated, they produce pro inflammatory cytokines [32, 33]. Cultured osteoblasts, like cells in vitro, secrete cytokines such as IL-1 and IL-6 [34-36]. It has been found that IL- 6 produced by osteoblasts may play a role in the activity of osteoclasts associated with bone resorption [37-39].

Previous investigations on bacterial arthritis induced by *S. aureus* and *S. agalactiae* have suggested that TNFα, interleukin1β, and IL-6 are associated with pathogenesis of bacterial arthritis [40, 41]. TNFα is known to be involved in osteoclastogenesis and stimulate osteoclast formation and differentiation [42]. Both IL1β and TNFα are thought to contribute directly to tissue damage by activation of osteoclasts which induce the release of tissue-damaging enzymes [43, 44]. In addition to stimulating resorption, it is known that IL1β and TNFα also inhibit bone formation in vitro [45-50]. IL1β expression was induced by all three bacterial infections with early induction by AB and KP, while PA infection induced IL1β at all time points [Table 8]. TNFα expression was also induced early for AB, and for KP at three time points. PA infection resulted in the up-regulation of TNFα at all time points [Table 8]. Previously, Bost et al [51] demonstrated that cultured murine or human osteoblasts express high levels of IL-6 following exposure to *S. aureus* by both gene expression and protein secretion. *Brucella* infection of osteoblasts also induced IL-6 secretion [52].

IL-6 expression was increased at the early time points for AB and KP. In addition, for PA, IL-6 was induced at 48 hours post infection. The neutrophil attractant chemokine IL-8 expression was significantly expressed for AB at 4 and 24 hours, while for KP it was also expressed at 48 hours. PA induced IL-8 at all time points [Table 8]. A recent study indicated that infection with *Chlamydia pneumoniae* induced the maximum expression of pro-inflammatory IL-8 (among other cytokines) at 72 hours post infection in SaOs2 osteoblast cell line [53]. The expression of IL-8 suggests that during infections with these pathogens neutrophils may play an important role in clearing the infection. The overall expression patterns of these chemokines/cytokines suggest that intracellular AB, KP and PA may elicit inflammatory responses in osteoblasts which in turn may also hinder the differentiation and maturation of osteoblasts.

We tested the supernates collected from the wells of the 3-D osteoblast scaffolds after infection with AB, KP and PA at 4, 24, 48 and 72 hours post infection on a multiplex Luminex cytokine/chemokine panel. The Luminex cytokine/chemokine panel consisted of the following: GMCSF, IFNg, IL-10, IL-12p40, IL-13, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL1β, MCP-1 and TNFα.

We detected the presence of IL-6 in the supernates of infected osteoblasts [Fig 9] which was in agreement with the gene expression data [table 8]. The pro-inflammatory cytokine IL-6 was detected in smaller quantities [Fig 9]. We also detected large amounts of IL-8 in the supernates [Fig 9]. Interestingly, even though IL1β and TNFα genes were induced during infection, we did not detect secreted IL1β or TNFα proteins to any of the 3 pathogens in the supernates of infected osteoblasts.

Milestone 2: Therapy with antimicrobial peptides

The second stage of the proposed study was to evaluate the activity of antimicrobial peptides on infected osteoblasts and study the therapeutic activities of these peptides. We tested the bactericidal activity of a single peptide, KSL-W, against AB and KP in the 3-D osteoblast model as it was not effective against PA.

We found that *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* may survive intra-cellularly in osteoblasts. If antibiotics or other therapeutics are administered, it may result

in survival of these pathogens intra-cellularly due to inadequate amounts of antibiotic or therapeutic that infiltrates osteoblasts.

A synthetic antimicrobial decapeptide KSL was identified [54]. KSL was reported to be a promising antimicrobial agent to be incorporated in chewing gum formulation as it prevented the development of oral biofilm and inhibit oral pathogens in vitro [55-57]. The stability of KSL and its analogs was determined [58] and KSL-W (KKVVFWVKFK-NH(2)), was selected as a candidate that could be used as an antiplaque agent in a chewing gum formulation [58]. As KSL-W is known to have bactericidal activity against *A.baumanni* and *K.pneumoniae* (personal communication with Dr. Leung), we tested its ability to treat infected osteoblasts in the 3-D scaffolds when infected with *A.baumannii* and *K pneumoniae*.

We determined the bactericidal activity of KSL-W peptide 1 hour post-treatment against extracellular bacteria compared with polymyxin B and untreated controls according to protocols. For AB, 100ug/ml of KSL-W was effective against extracellular bacteria present in the medium of the 3-D osteoblast scaffolds compared to the untreated controls. Polymyxin B (100ug/ml) treatment was very effective in killing extracellular bacteria by 1 hour resulting in 0 CFU. When intracellular AB was determined, both untreated and KSL-W treated cells had similar numbers of intracellular AB, while polymyxin B treated osteoblasts had less intracellular bacteria [Fig 10 a]. Similarly, for KP, treatment with KSL-W resulted in less extracellular bacteria compared to untreated control.

Again, polymyxin B treatment was very effective against extracellular KP after 1 hour treatment. Intracellular KP was similar to untreated cells, while polymyxin treated cells yielded less bacteria [Fig 10 b]. Most probably, the reason for higher numbers of intracellular bacteria for AB and KP in both untreated and KSLW treated cells is continuous re-infection from extracellular bacteria in the medium. PA was not sensitive to the killing of KSL-W (data not shown).

KEY RESEARCH ACCOMPLISHMENTS

- Establishment of a 3-D collagen scaffold with primary human osteoblasts to study their differentiation by gene expression.
- Developed a working 3-D model of primary osteoblasts on a collagen scaffold to study the interaction of osteoblasts and bacteria by gene expression.
- Significant finding - Three Gram negative pathogens associated with osteomyelitis in war wounds, namely, *Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Klebsiella pneumoniae* may survive intra- cellularly in osteoblasts. Findings indicate that *Pseudomonas* and *Klebsiella* sp are more invasive to osteoblasts than *Acinetobacter sp.* and may survive with higher numbers of intracellular bacteria.

REPORTABLE OUTCOMES

1. A working 3-D in vitro model of primary human osteoblasts on collagen scaffolds has been developed and established to perform infection of osteoblasts with bacteria.
2. This model may be utilized to study gene expression of osteoblasts and long term cultures of osteoblasts. It may also be used to study therapy with new antimicrobials.
3. Gram negative *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* associated with osteomyelitis can survive intracellularly in osteoblasts. Once intracellular, they may have the ability to reinfect new osteoblasts.

Manuscripts, abstracts, presentations

1. Presented ongoing work at the wound symposium in May 2010 in San Antonio, Texas
2. Manuscript in preparation

Challenges in the research project

The main challenge to this work was working with primary osteoblasts. Work with primary osteoblasts is challenging as they grow very slow, and have limited ability to divide.

The proposed work was to test antimicrobial peptides to treat infected osteoblasts. The findings from this work suggest that all three species of bacteria can survive intra-cellularly in osteoblasts. The tested peptide KSL-W does not penetrate eukaryotic cell membranes, and future studies are needed to modify the peptide or encapsulate the peptide to reach the intracellular bacteria in osteoblasts.

CONCLUSION

An important finding from this study was that three Gram negative bacteria associated with war related osteomyelitis, namely, *A.baumannii*, *K.pneumoniae* and *P.aeruginosa*, may survive intra-cellularly in osteoblasts. Once they survive as intracellular pathogens, they may have the ability to re-infect new osteoblasts.

This work resulted in the development of a working in vitro 3-D model comprised of primary human osteoblasts on a collagen scaffold. This model may be used to study the interaction of osteomyelitis related pathogens with osteoblasts. This model may also be a more "complete" system if one can add CD14 positive monocytes (which can differentiate into osteocytes). Also, it can be utilized for long term culture as a 3-D osteoblast-osteocyte model. Utilizing this model, infection of osteoblasts can be performed, cells can be harvested, and RNA can be extracted and used for gene expression studies. The 3-D model can be used to evaluate novel antimicrobials against osteomyelitis associated pathogens.

Post infection, the expression of several genes related to osteoblast differentiation, maturation and inflammation was investigated. Infection with AB resulted in osteoblast marker genes ALP and BGLAP being either reduced or expression unchanged, suggesting that osteoblast differentiation may not be optimal. Another important gene related to osteoblast differentiation, Dlx5, was either down-regulated or had no significant changes when infected with AB, KP or PA. The transcription factor Twist1 needs down-regulation for osteoblast differentiation, yet we saw up-regulation when infected with AB and PA, and no significant change with KP. Cell adhesion genes were also impacted by infection as N-cadherin (CDH2) was down-regulated or not significant at the later time points except for the initial up-regulation at 4 hours for KP and PA. Interestingly, the growth factor gene BMP2 was up-regulated only for AB, and BMP7 was up-regulated for AB and KP. Infection with PA did not result in significant changes to either BMP2 or 7.

The master transcription factor Runx2, which is the activator of osteoblast differentiation, was not significantly different from the uninfected controls for AB and KP and increasing for PA at 72 hours. As the expression of several other genes also may depend on Runx2, it could contribute to the lack of

expression observed. Overall, our results suggest that even though osteoblasts harbor low numbers of these intracellular pathogens, it may result in a delay in osteoblast maturation and well-being at 3 days post infection. This is indicated by the differential expression of several genes associated with osteoblast maturation such as ALP, CDH2, TWIST1 and Stat1.The long term effects of such infections on the bone cells are unknown.

In addition, AB infection elicited an early inflammatory response with IL1β, IL-8, IL-6 and TNFα expression. KP infection was similar, except IL-8 and TNFα were induced at 48 and 72 hours post infection respectively. PA infection resulted in a more robust expression of these genes across the time points. The secreted IL-6 by osteoblasts suggests that IL-6 may contribute towards development of protective responses. IL-6 may also result in harmful effects on the bone such as inflammatory damage and bone resorption. The secretion of high levels of neutrophil attractant IL-8 suggests that neutrophils may also be important towards protective responses and clearing the infection. We found some differences in the expression of IL-6 and IL-8 gene expression secreted amounts detected. We believe that if our independent sample numbers were increased to at least 6 or more instead of 3, these differences can be minimized. It is recommended that for future work, sample size needs to be increased. These pro-inflammatory responses in osteoblasts suggest that intracellular infection with these bacteria does result in changes to the bone cells which may affect their differentiation and maturation processes.

Treatment of osteoblast infections with peptide KSL-W was tested. KSL-W was effective in decreasing the extracellular bacterial load in the media with scaffolds for both AB and KP. Intracellular bacteria were not reduced significantly by KSL-W treatment. As appropriate, it is recommended that therapies should be focused towards the eradication of both extra and intracellular bacteria. For example, cell penetrating peptides should be tested against these bacteria. An antimicrobial peptide formulation in nanoparticle encapsulation may be more appropriate for treatment of intracellular AB, KP and PA infections of the osteoblasts.

For future work, in order to improve the 3-D osteoblast model, additional cells such as monocytes may be included in this system as monocytes can differentiate to osteoclast cells which are the other major cell type in bone tissue. This would further improve the in vitro model towards a more organotypic

tissue model of the bone environment by the presence of osteocytes together with osteoblasts. It would then be possible to study the interaction of these pathogens with both major bone cell types. With both types of cells included in the 3-D model, one can also study the effect of infection in osteoblast mineralization which occurs after long term culture. The outcome of these findings may contribute to accelerated treatment options and management of osteomyelitis due to drug resistant Gram negative bacteria.

“So what?”

Osteoblasts and other cell types in vivo have a typically 3-D structure from which they are able to migrate, attach and proliferate. To study osteoblasts in vitro, a natural matrix such as collagen to act as support for seeded osteoblasts is vital. In order to study the interaction of bacteria with osteoblasts, a 3-D model of infection is essential as it is known that osteoblasts in a 3-D environment are more physiologically relevant. The developed model is a relatively straightforward osteoblast 3-D model that can be utilized to study diverse aspects of osteoblast interactions.

The important finding from the present work is that *Acinetobacter baumannii, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* have the ability to reside as intracellular pathogens in osteoblasts. The intracellular survival of these pathogens may also affect osteoblast maturation and differentiation, and could potentially delay bone wound healing. Most antibiotics used to treat osteomyelitis do not penetrate osteoblasts. Antimicrobials such as peptides that can penetrate the host cells should be developed to eradicate intracellular pathogens. These findings could potentially change treatment options for osteomyelitis in soldiers infected with these Gram negative pathogens.

SUPPORTING DATA:

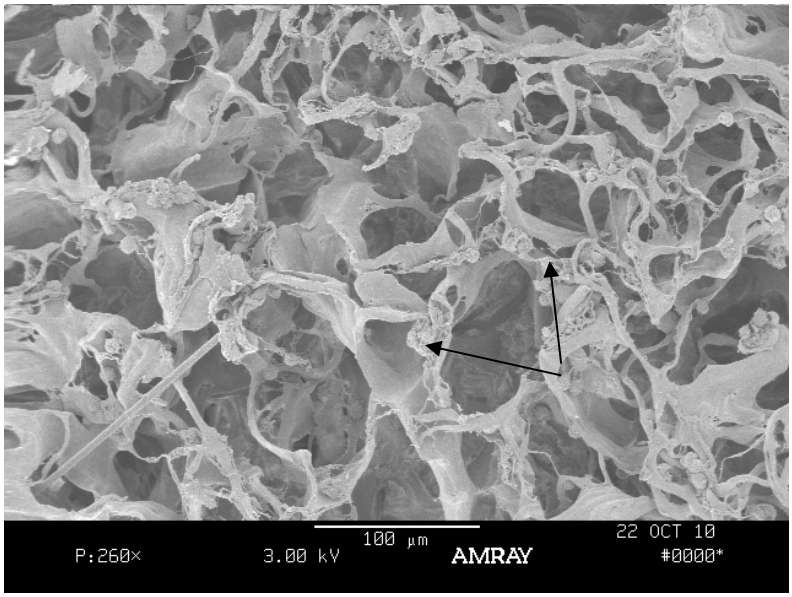


Fig 1. Scanning Electron Micrograph of the scaffold used for this study seeded with SaOs2 osteoblasts (mag. 160X). The cells are seen spreading over the surface of the scaffold with extracellular matrix formation (arrow).

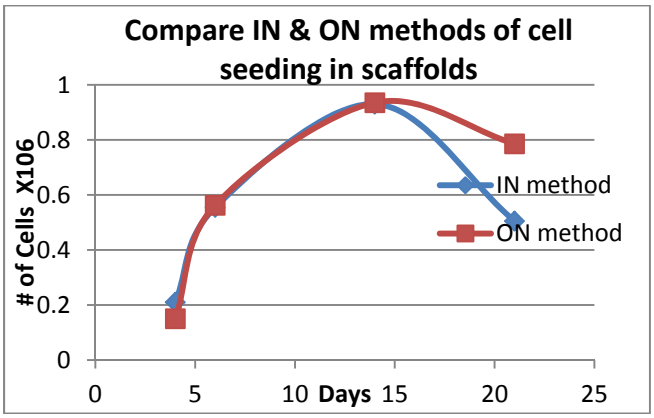


Fig 2. Comparison of two different methods used for cell seeding into the scaffolds for the development of the 3-D osteoblast model. Y axis indicates the number of cells attached to the scaffold over time using IN and ON methods. Data are means of triplicate for each time point.

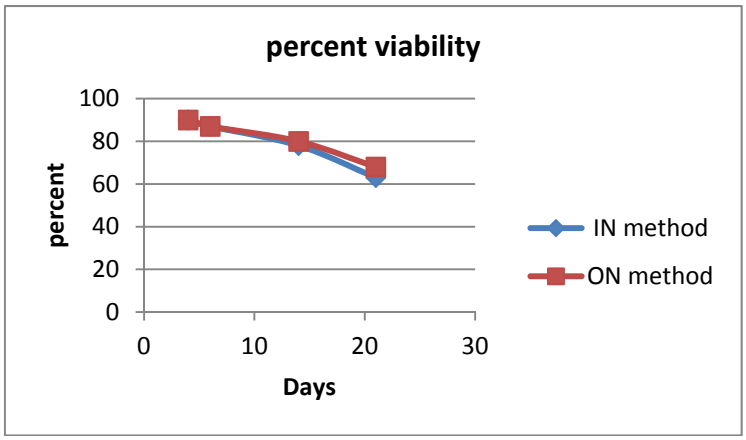


Fig 3. Osteoblast viability detected over time when seeded on scaffolds. Comparison of the two methods shows that there is no difference in viability of cells seeded with the IN or ON method. Data represents a single experiment with means of triplicate scaffolds for each time point.

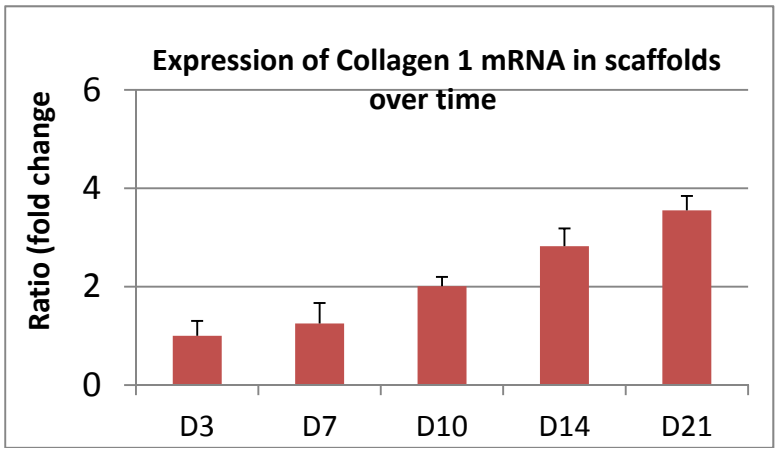


Fig 4. Expression of collagen 1 in SaOS2 cells attached to the collagen scaffolds over time. Data are means from a single experiment performed in triplicate. The ability to extract total RNA for the work proposed was investigated in order to perform gene expression work. From each scaffold, after collagenase digestion, an average of 600ng (260/280 ratio 1.9) of total RNA was extracted from osteoblasts by using RNAzol RT following manufacturer's instructions. Once cells were isolated, Real Time PCR was performed on SaOs2 cells with SYBR green.

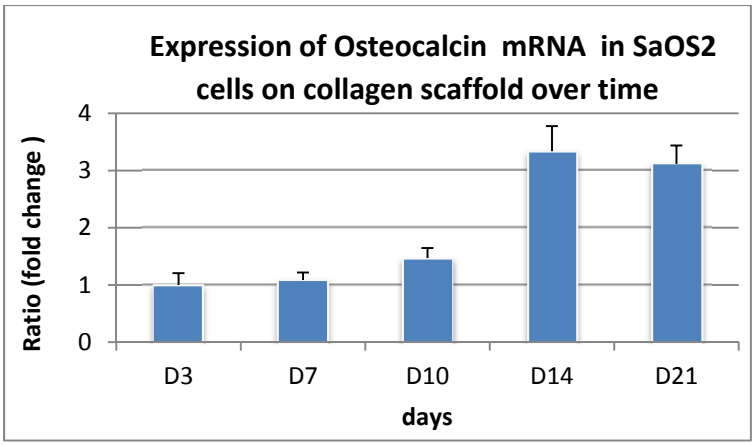


Fig 5. Expression of Osteocalcin (BGLAP), a marker for osteoblast maturation in SaOS2 cells attached to the collagen scaffolds over time. Data are means from a single experiment performed in triplicate.

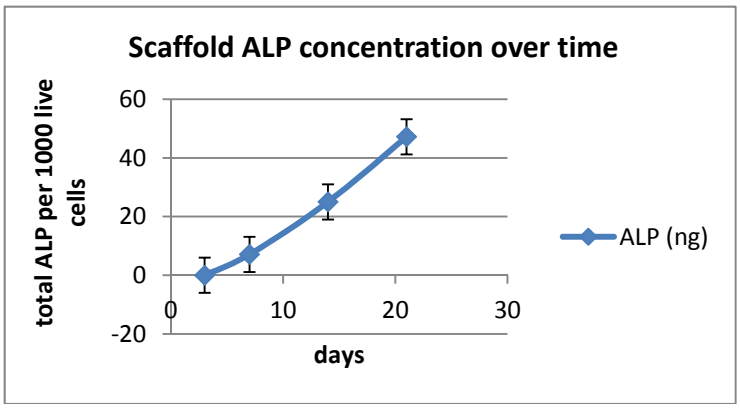
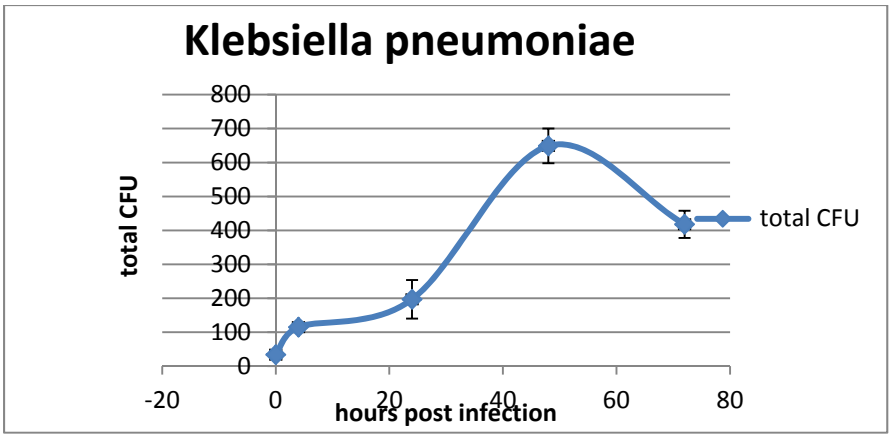
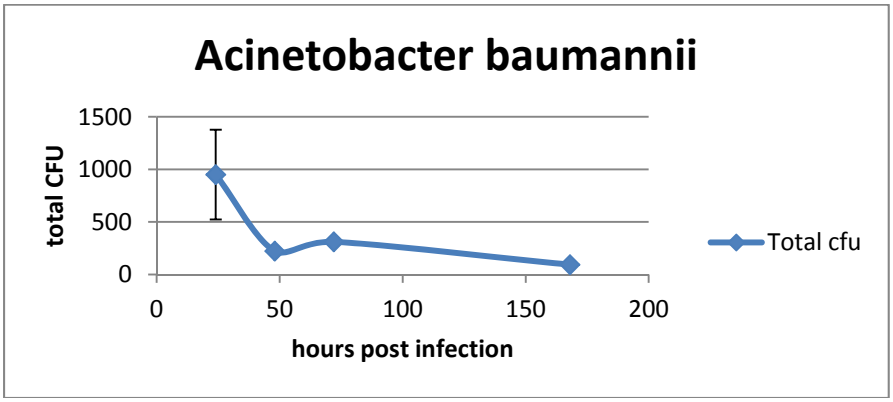


Fig 6. The detection of alkaline phosphatase (ALP) in osteoblasts in the 3-D scaffold. The quantity of ALP increased in SaOs2 cells on the scaffold over time. Data are means of a single experiment performed in triplicate. To assay the presence of alkaline phosphatase in the cells, cells were lysed in the lysis buffer (0.2% v/v TritinX-100,10mM Tris (PH 7) and 1m MEDTA). Following centrifugation, equal volumes of cell lysate were assayed for the presence of alkaline phosphatase using an Alkaline Phosphatase Assay Kit (Anaspec Co). The colorimetric reactions, executed in the linear range, were determined after 15 minutes and the absorbance was measured at 405 nm. Background absorbance was subtracted from the total value. Alkaline Phosphatase (ALP) was expressed as the quantity per 1000 live cells, at each time point.





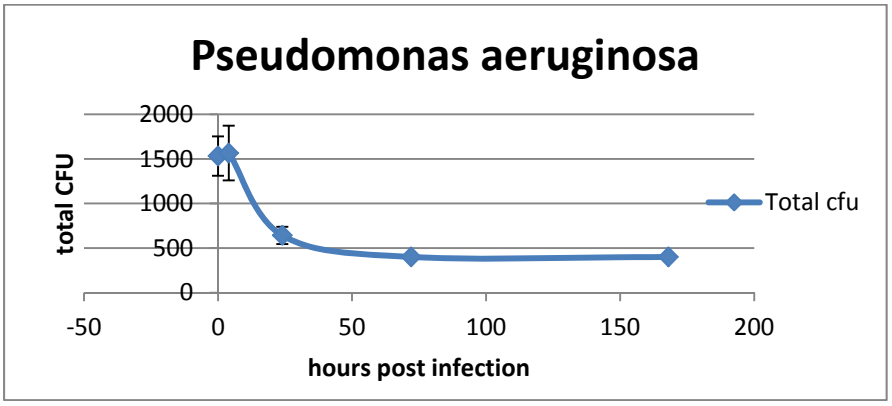


Fig 7. The detection on intracellular *Acinetobacter baumannii*, *Klebsiella pneuminiae* and *Pseudomonas aeruginosa* from osteoblasts grown on collagen scaffolds in the 3-D model.

	Number of intracellular bacteria (CFU/well)					
	Lysed (control)	Trypsinized cells	Lysed (control)	Trypsinized cells	Lysed (control)	Trypsinized cells
time	*Acinetobacter baumannii*		*Klebsiella pneumoniae*		*Pseudomonas aeruginosa*	
0	480	360	$3.6x10^3$	$1.8x10^3$	$21x10^3$	$13.5x10^3$
24	720	360	$4.5x10^3$	$3x10^3$	$3.9x10^3$	$6.9x10^2$
72	$3x10^3$	$2x10^3$	$8.2x10^3$	$3.9x10^3$	$2.7x10^3$	$8.4x10^2$

Table 1. The recovery of intracellular bacteria from cell lysis vs. plating of whole cells.

Table 1 shows the number of intracellular bacteria recovered when monolayer SaOs2 cells were infected with AB, KP and PA at 0, 1 and 3 days for the reinvasion assay. The method of recovery was compared between lysis of cells with water and infected cells trypsinized and plated out. More bacteria were recovered when cells were lysed. Results are from a single experiment which was repeated at least three times.

Intracellular CFU after reinfection	Number of intracellular bacteria (CFU/well)
Acinetobacter baumannii	$3.15x10^3$
Klebsiella pneumoniae	$18.9x10^3$
Pseudomonas aeruginosa	$38.4x10^3$

Table 2. The ability of intracellular bacteria recovered from trypsinized osteoblasts to re-infect healthy osteoblasts. Bacteria recovered from infected osteoblasts (SaOs2) after trypsinization was used to re-infect fresh cells. The extracellular bacteria were killed with polymyxin B, and the intracellular bacteria were recovered after one hour. Results are from a single experiment which was repeated at least three times.

GENE SYMBOL	GENE REFSEQ #
ALP	NM_000478
VDR	NM_000376
BGLAP	NM_199173
CD11	NM_001797
FOXC2	NM_005251
RUNX2	NM_004348
ATF4	NM_001675
STAT1	NM_007315
OSTERIX (SP7)	NM_152860
Dlx3	NM_005220
Dlx5	NM_005221
C-FOS	NM_005252
Msx2	NM_002449
Twist1	NM_000474
HEY1	NM_012258
CDH4	NM_001794
CDH2	NM_001792
IBSP	NM_004967
LEF1	NM_016269
BMP2	NM_001200
BMP7	NM_001719
BETA ACTIN	NM_001101
GAPDH	NM_002046

Table 3**.** The selected gene list for RT^2 Profiler^{TM} custom PCR array for osteoblast infections.
Total RNA from primary osteoblasts infected with AB, KP and PA were used to probe the arrays at t=4, 24, 48 and 72 hours post infection. An uninfected control was included at each time point. The PCR Arrays consisted of sets of optimized real-time PCR primer assays with osteoblast differentiation/maturation focused genes as well as appropriate RNA quality controls on 96-well plate format. The RT PCR array performed gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray. The RT PCR arrays were completed following the manufacturer's instructions (QIAGEN RT 2 Profiler ^{TM}). Briefly, total RNA (500ng) was converted to cDNA according to the manufacturer's protocol (Qiagen Inc). cDNA was added to RT 2 qPCR master mix, and

was added to the 96 well custom array which included the appropriate primers for each gene. PCR arrays were then run in an ABI 7000 instrument. Data was uploaded according to instructions. The mRNA expression levels obtained for each gene were normalized to the mean expression of GAPDH and beta actin housekeeping genes by using the following equation: relative mRNA expression = 2 −(Ct of test gene −Ct of mean housekeeping genes) (where Ct is the threshold cycle). The integrated web-based software package for the PCR Array System provided by Qiagen (SA BioSciences) automatically performed all ΔΔC t based fold-change calculations from the uploaded raw threshold cycle data.

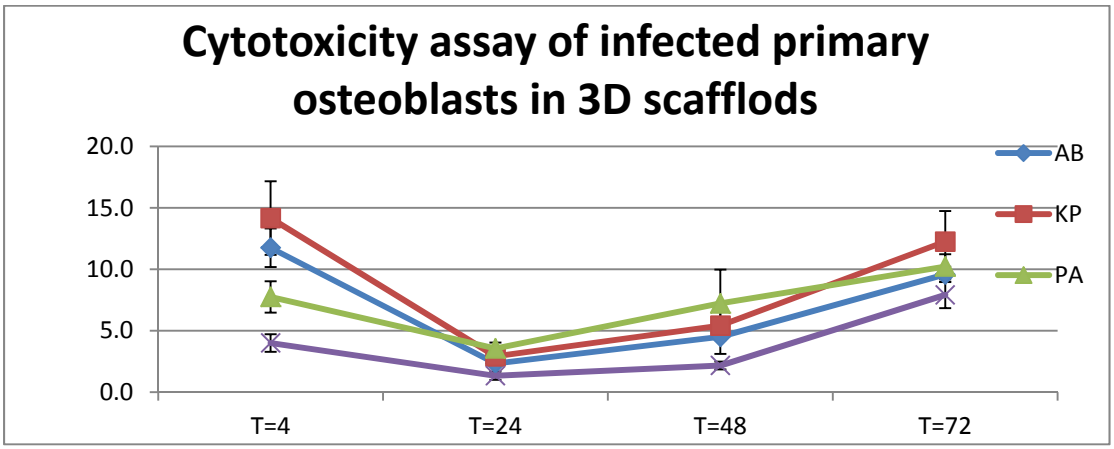


Fig 8**.** LDH measurements of supernatants from primary osteoblasts infected with bacteria over time. Cytotoxicity of primary osteoblasts on 3-D scaffolds when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) were compared to uninfected controls (n=3) at time points of 4, 24, 48 and 72 hours post infection. LDH released from the adhered cells in scaffolds was quantified. Y axis=Measurements of units/LDH/ml.

Gene	4 hr	24 hr	48hr	72hr	Bacteria
ALP	-3.66	1.75	-3.16	-1.35	AB
	ns	-2.47	ns	ns	KP
	ns	ns	-6.40	-2.18	PA
BGLAP	-1.83	ns	ns	ns	AB
(osteocalcin)	ns	-1.82	ns	ns	KP
	ns	ns	-2.54	ns	PA

Table 4. Temporal expression of two osteoblast marker gene expression in primary human osteoblasts in 3-D scaffold and infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values represent mean fold changes with up-regulation as positive and down-regulation as a negative value. Significant ($p>.05$) fold changes (Students t-test) compared to uninfected control (n=3).ns denotes not significant.

GENE	4 hr	24 hr	48hr	72hr	Bacteria
STAT1	ns	3.99	2.68	ns	AB
	2.26	2.25	3.58	ns	KP
	ns	3.17	1.86	2.65	PA
SP7 (Osterix)	ns	3.10	ns	ns	AB
	ns	ns	ns	2.28	KP
	ns	ns	ns	ns	PA
Dlx3	-4.41	ns	ns	ns	AB
	-4.53	-1.95	ns	ns	KP
	ns	-2.35	-1.53	ns	PA
Dlx5	-1.99	ns	ns	ns	AB
	ns	ns	ns	ns	KP
	ns	ns	-2.98	-2.62	PA
Twist-1	ns	4.36	ns	ns	AB
	ns	ns	ns	ns	KP
	2.40	ns	2.71	1.90	PA
c-fos	-2.07	3.66	2.12	2.03	AB
	ns	ns	ns	ns	KP
	ns	ns	ns	2.07	PA

Table 5. Temporal gene expression of osteoblast transcription factors in primary human osteoblasts in the 3-D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values are significant (p>.05) fold changes (Students t-test) compared to housekeeping gene value=1 (n=3).ns denotes not significant.

GENE	4 hr	24 hr	48hr	72hr	Bacteria
BMP2	ns	18.08	5.06	ns	**AB**
	ns	ns	ns	ns	**KP**
	ns	ns	ns	ns	**PA**
BMP7	ns	ns	ns	9.73	**AB**
	2.20	ns	ns	8.14	**KP**
	ns	ns	ns	ns	**PA**

Table 6. Temporal gene expression of bone morphogenic proteins BMP2 and 7 in primary human osteoblasts in the 3-D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values are significant (p>.05) fold changes (Students t-test) compared to housekeeping gene values=1 (n=3).ns denotes not significant.

GENE	4hr	24hr	48hr	72hr	Bacteria
CDH2	ns	ns	-2.57	ns	AB
	2.36	ns	ns	-3.89	KP
	2.49	ns	ns	-1.78	PA
CDH4	ns	ns	ns	ns	AB
	ns	-2.55	ns	ns	KP
	ns	ns	ns	ns	PA

Table 7. Temporal expression of cell-adhesion related genes in primary human osteoblasts in the 3D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values are significant (p>.05) fold changes (Students t-test) compared to housekeeping gene value=1 (n=3).ns denotes not significant.

GENE	4 hr	24 hr	48hr	72hr	Bacteria
IL1β	17.46	4.47	ns	ns	AB
	58.59	5.92	ns	ns	KP
	10.73	41.82	25.04	44.99	PA
IL-8	29.52	18.35	ns	ns	AB
	27.16	46.47	47.29	ns	KP
	13.79	23.68	24.41	46.58	PA
IL-6	5.03	21.17	ns	ns	AB
	19.26	25.25	ns	ns	KP
	21.98	34.47	24.48	ns	PA
TNFα	3.71	1.94	ns	ns	AB
	26.43	2.83	ns	2.40	KP
	29.69	6.85	5.55	7.55	PA

Table 8. Temporal expression of cytokines/chemokine genes in primary human osteoblasts in the 3-D scaffold when infected with *Acinetobacter baumannii* (AB), *Klebsiella pneumoniae* (KP), *Pseudomonas aeruginosa* (PA) compared to uninfected controls. Values are significant (p>.05) fold changes (Students t-test) compared to housekeeping gene value=1 (n=3).ns denotes not significant.

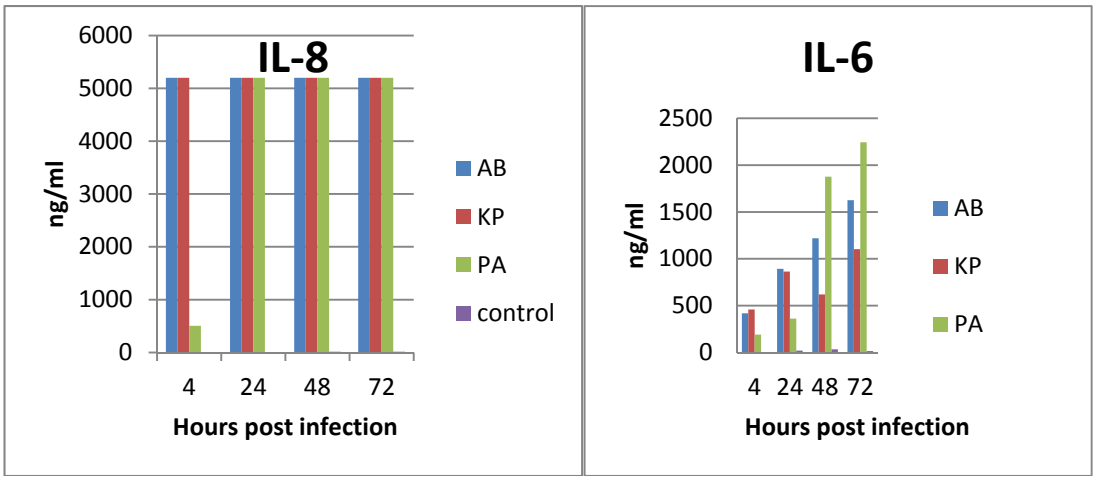


Fig 9. Cytokines detected in the supernates of infected osteoblasts in the 3-D scaffolds. Data represents a single experiment that was repeated at least three times. Cytokine detection was performed on supernates collected from the wells of 3-D osteoblast scaffolds either infected with AB, KP or PA or uninfected samples. Supernates were collected at 4, 24, 48 and 72 hours post infection. Multiplex ELISA assays were performed on the samples according to the manufacturers instructions (Luminex Inc).

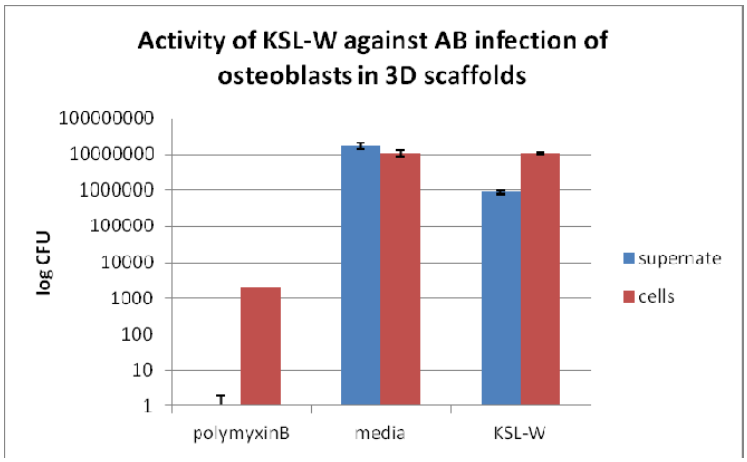


Fig 10 (a)

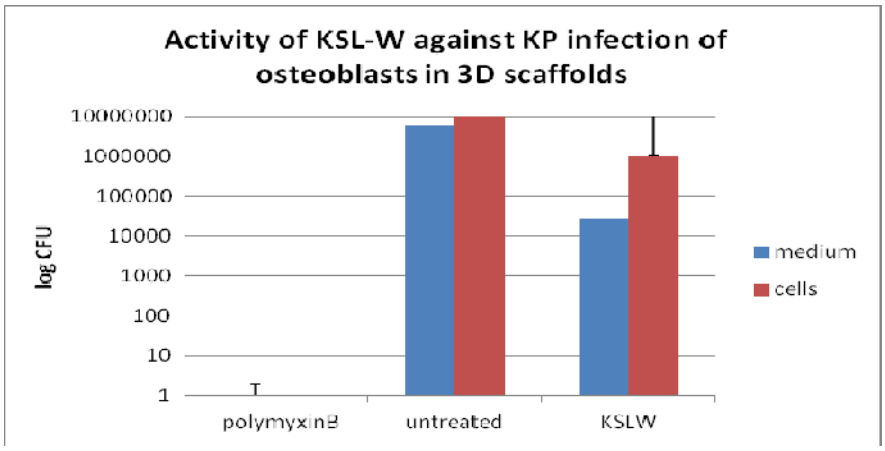


Fig 10 (b)

Fig 10. The activity of KSL-W antimicrobial peptide against AB and KP.
KSL-W (100ug/ml) was added for treatment of infected osteoblasts (in scaffolds), polymyxin B (100ug/ml) for 1 hour post infection; AB infection (a) and KP infection (b). Controls included polymyxin B (100ug/ml) and untreated osteoblasts. This experiment represents typical results from a single experiment with triplicate wells/condition. This was repeated independently at least two times.

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